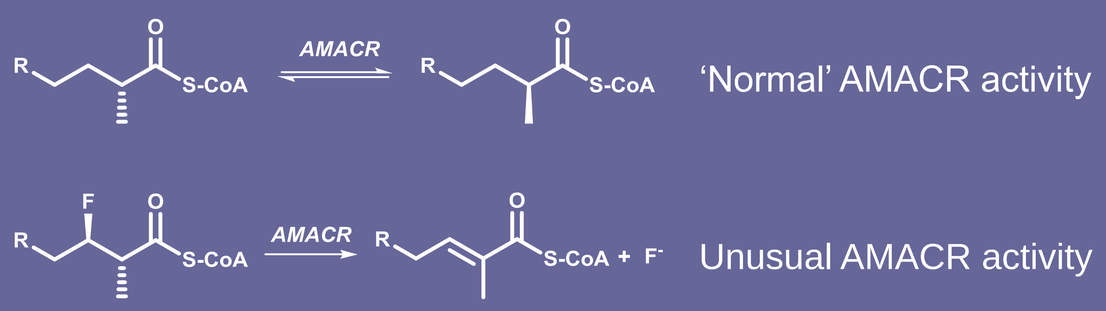
A Convenient Colorimetric Assay for α-Methylacyl-CoA Racemase (AMACR; P504S) And Testing Of Inhibitors

Maksims Yevglevskis,[a] Guat L. Lee,[a] Yoana D. Petrova,[a] Amit Nathubhai,[a] Tony D. James,[b] Michael D. Threadgill,[a] Timothy J. Woodman[a] and Matthew D. Lloyd[a,‡]

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, U.K. ‡email: M.D.Lloyd@bath.ac.uk
Department of Chemistry, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

Introduction

Branched-chain fatty acids are common in the diet and similar structures are found in medicines such as Ibuprofen and related drugs. Metabolism of branched-chain fatty acids requires that the centres bearing the methyl groups possess *S*-stereochemical configuration, but those with *R*-configuration are produced in the body and are found in the diet. Ibuprofen and related drugs require *S*-configuration for their anti-inflammatory properties, but these drugs are usually given as a mixture of *R*- and *S*-enantiomers. The enzyme α-methylacyl-CoA racemase (AMACR) catalyses *R*- to *S*- conversion of 2-methylacyl-CoA derivatives of fatty acids (Scheme 1) enabling β-oxidation. Similarly, acyl-CoA derivatives of Ibuprofen and similar drugs are converted, resulting in pharmacological activation.[1,2]

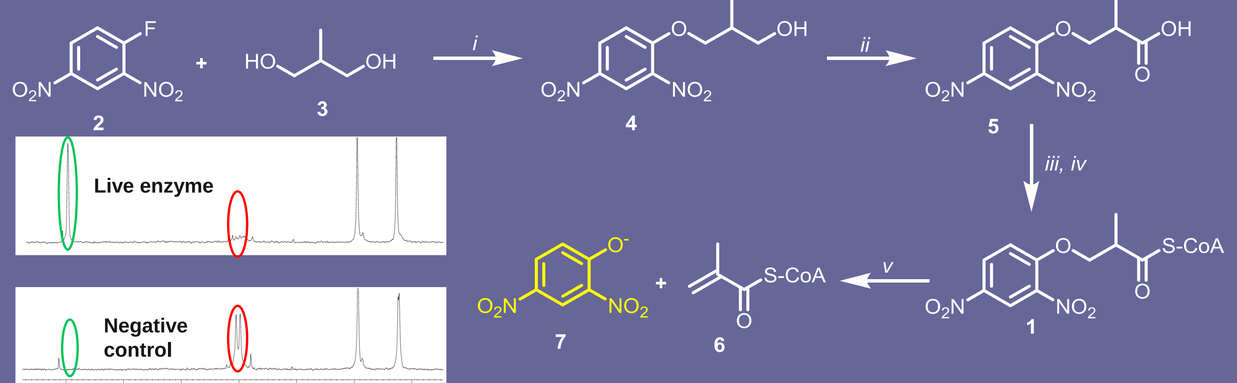


Scheme 1: AMACR catalysed 'racemisation' and elimination reactions.

AMACR levels are increased in all prostate cancers, some colon cancers and other cancers.[1-3] In prostate cancer, higher AMACR levels result in higher proliferation rates[4] and androgen-independent growth[5] and AMACR is recognised as a novel drug target. However, few inhibitors have been identified, largely due to the difficulties in measuring enzyme activity which makes it difficult to quantify drug potency.[1] AMACR catalyses the irreversible elimination of hydrogen fluoride from 3-fluoro-2-methylacyl-CoA substrates (Scheme 1),[6] but translating this reaction to a convenient colorimetric or fluorometric assay has proven difficult.[3] 4-Nitrophenol derivatives are commonly used as colorimetric substrates for enzymes. This study reports the synthesis of a 2,4-dinitrophenol-containing AMACR substrate and the characterisation of known AMACR inhibitors using a convenient colorimetric microtitre plate assay.

Results and Discussion

2,4-Dinitrophenol is fully ionised at neutral pH giving a yellow colour and has a similar *p*Ka to HF, which is eliminated from known AMACR substrates. Therefore an acyl-CoA derivative **1** containing 2,4-dinitrophenol was investigated. Reaction of **2** with alcohol **3** to give **4** followed by oxidation gave the racemic acid **5,** which was converted to the desired substrate **1** (Scheme 2). Incubation of **1** with recombinant human AMACR 1A resulted in formation of unsaturated product **6** and 2,4-dinitrophenol **7** resulting in a yellow colour.



Scheme 2: Synthesis of novel substrate **1** and reaction with AMACR. *Reagents & conditions*: i. Na metal; ii. Jones oxidation; iii. CDI, DCM; iv. CoA-SH, $NaHCO_{3\ aq}$./THF (1:1); v. NaH_2PO_4-NaOH, pH 7.4, *ca*. 77% ^2H_2O.

AMACR was active around neutral pH and retained full activity in the presence of 8% (v/v) DMSO. Kinetic analysis of substrate 1 showed that Michaelis-Menten kinetics were observed, with the following parameters: K_m = 56 ±4.5 μM; V_{max} = 112 ± 4 nmol.min.$^{-1}$mg^{-1}; k_{cat} = 0.088 s^{-1}; k_{cat}/K_m = 1571 s^{-1} M^{-1}. This shows that substrate **1** is converted with ~44% of the efficiency of 3-fluoro-2-methyldecanoyl-CoA and was significantly more efficient than 'racemisation' of 2-methyldecanoyl-CoA (as judged by k_{cat}/K_m).[6]

The known inhibitor Rose Bengal[7] was tested to validate the method for characterisation of inhibitors (Figure 1). A dose-response curve was efficiently produced using a microtitre plate assay.

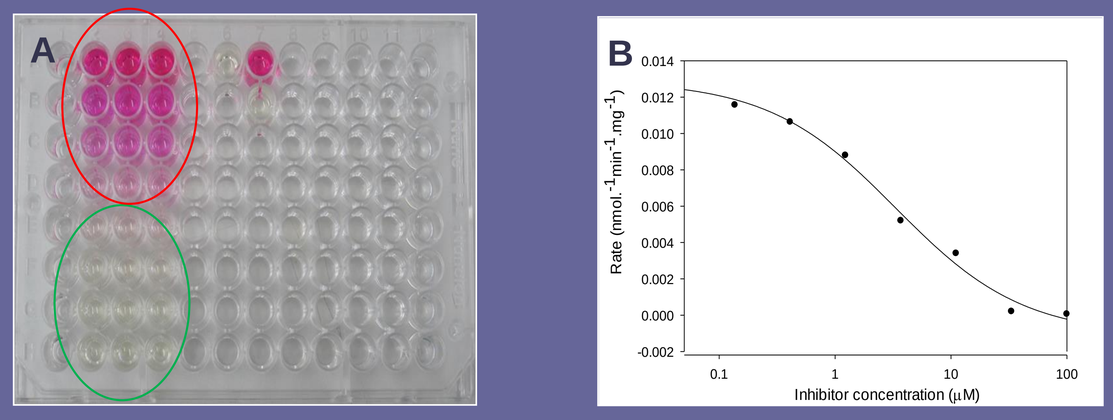


Figure 1: AMACR inhibition assay using Rose Bengal as an inhibitor. A. 96-Well plate showing colour change; B. Dose-response curve for Rose Bengal.

A number of other known AMACR inhibitors and substrates were tested using a dose-response curve at a fixed substrate concentration of 40 μM. Ibuprofenoyl-CoA and related compounds are known substrates and should behave as competitive inhibitors. All of these compounds inhibited the enzyme with IC_{50} values of *ca.* 400-800 nM. 2-Methyldecanoyl-CoA also inhibited the reaction, and was *ca.* 3x more potent than decanoyl-CoA. Inhibition was decreased in acyl-CoA esters with shorter alkyl chains. The best acyl-CoA inhibitor was *N*-dodecyl-*N*-methylcarbamoyl-CoA,[8] which was ~1000 x more potent than the other acyl-CoA inhibitors (as judged by IC_{50} values). The non-specific protein modifying reagents reported by Wilson *et al*.[7] also inhibited the enzyme; in contrast to previous reports Ebselen oxide behaved as a time- and concentration-dependent inactivator with a rate constant of 116 M^{-1} s^{-1}.

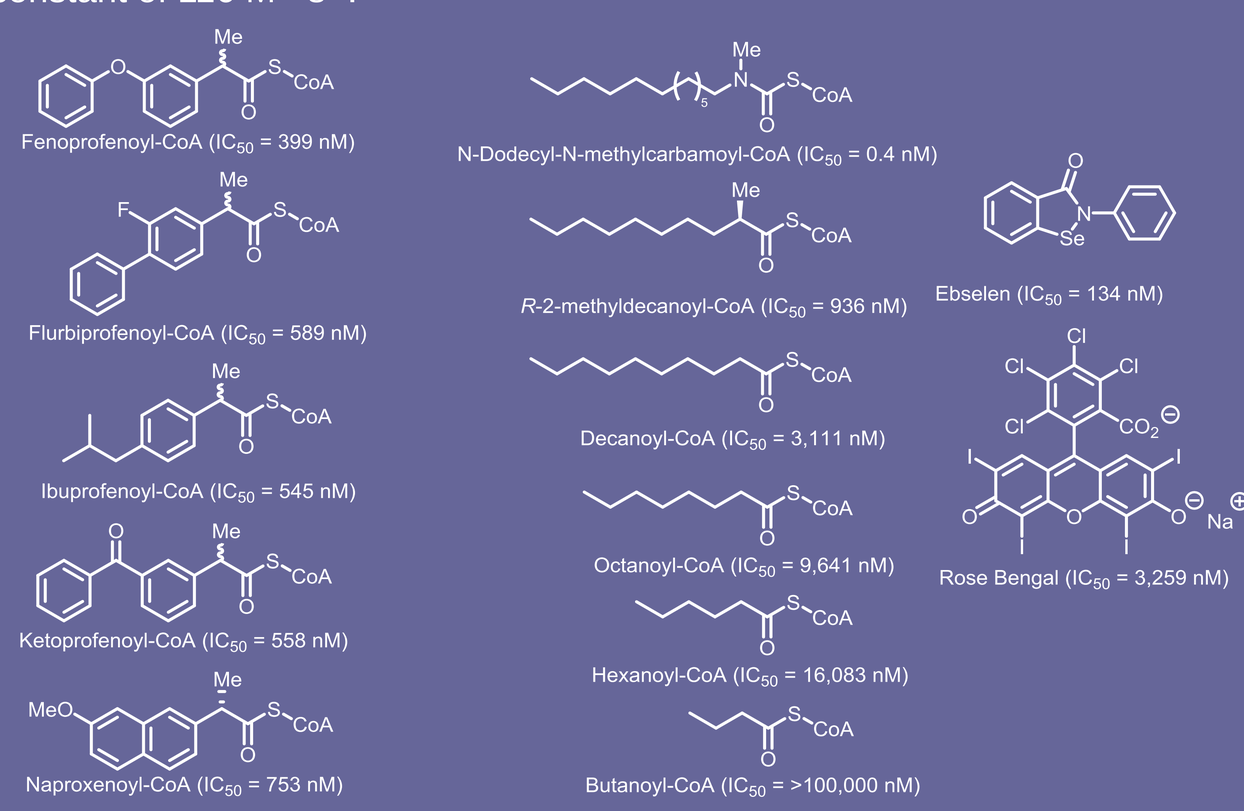


Figure 2: Selected acyl-CoAs and protein modifying agents shown to inhibit the conversion of substrate **1** to **6** and **7** by AMACR using the colorimetric assay.

Conclusions

The colorimetric substrate **1** provides a convenient method for assaying AMACR and determining the behaviour and potency of inhibitors. AMACR is a promising drug target for prostate and other cancers, but until now it has been under-exploited because of the difficulties in determining enzyme activities. Inhibitors previously reported in the literature are largely limited to rationally designed acyl-CoA esters, which do not comply with Lipinski guidelines.[9] This new assay will facilitate the testing and development of drugs by structure-based design, rational design and lends itself to screening approaches. The latter should allow identification of inhibitors with good drug-like properties.

Acknowledgements

This work was funded by Prostate Cancer UK (S10-03 and PG14-009), a University of Bath Overseas Research Studentship, BBSRC and Shandong-Bath undergraduate exchange studentships.

References

1. M. D. Lloyd, M. Yevglevskis, G. L. Lee, P. J. Wood, M. D. Threadgill and T. J. Woodman, *Prog. Lipid Res.*, 2013, **52**, 220-230.
2. M. D. Lloyd, D. J. Darley, A. S. Wierzbicki and M. D. Threadgill, *FEBS J.*, 2008, **275**, 1089-1102.
3. M. Yevglevskis, G. L. Lee, J. Sun, S. Zhou, X. Sun, G. Kociok-Köhn, T. D. James, T. J. Woodman, and M. D. Lloyd, *Org. Biomol. Chem*., DOI: 10.1039/c5ob01541c.
4. S. Zha, S. Ferdinandusse, S. Denis, R. J. Wanders, C. M. Ewing, J. Luo, A. M. De Marzo and W. B. Isaacs, *Cancer Res.*, 2003, **63**, 7365-7376.
5. K. Takahara, H. Azuma, T. Sakamoto, S. Kiyama, T. Inamoto, N. Ibuki, T. Nishida, H. Nomi, T. Ubai, N. Segawa and Y. Katsuoka, *Anticancer Res.*, 2009, **29**, 2497-2505.
6. M. Yevglevskis, G. L. Lee, M. D. Threadgill, T. J. Woodman and M. D. Lloyd, *Chem. Commun.*, 2014, **50**, 14164-14166.
7. B. A. P. Wilson, H. Wang, B. A. Nacev, R. C. Mease, J. O. Liu, M. G. Pomper and W. B. Isaacs, *Mol. Cancer Therapeut.*, 2011, **10**, 825-838.
8. A. J. Carnell, R. Kirk, M. Smith, S. McKenna, L.-Y. Lian and R. Gibson, *ChemMedChem*, 2013, **8**, 1643-1647.
9. C.A. Lipinski., F. Lombardo, B. W. Dominey, R. J. Feeney, *Adv. Drug Deliv. Rev*., 2012, **64S**, 4-17.